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THE ISOPROSTANES: UNIQUE BIOACTIVE PRODUCTS OF LIPID PEROXIDATION

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ABBREVIATIONS

PG	prostaglandin	Ms	mass spectrometry
Isop	isoprostane	Se	selenium
GC	gas chromatography		

I. INTRODUCTION

Free radicals, principally derived from oxygen, have been implicated in the pathophysiology of a wide variety of human diseases including cancer, atherosclerosis, neurodegenerative disorders and even the normal aging process.[1–4] Definitive evidence for this association is often lacking, however, because of recognized shortcomings with methods to assess oxidative stress status *in vivo*.[5]

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One of the well recognized targets of oxidant injury is peroxidation of lipids. In 1990, we reported the discovery that a series of prostaglandin (PG)-like compounds are produced by the free radical catalysed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme, which had heretofore been considered obligatory for endogenous prostanoid synthesis.[6] Since that time, we have accumulated a large body of evidence indicating that quantification of these unique products of lipid peroxidation, now termed isoprostanes (IsoPs), provides a reliable marker of oxidant injury both *in vitro* and *in vivo*. Further, at least two of these compounds possess potent biological activity and thus may participate as mediators of oxidant injury. It is the purpose of this review to summarize the status of our knowledge regarding the IsoPs.

II. HISTORICAL BACKGROUND

As early as the 1960s, it was shown that PG-like compounds could be formed by the autoxidation of purifed polyunsaturated fatty acids.[7–10] Elegant studies carried out by Pryor, Porter, and others led to a proposed mechanism by which these compounds were generated via bicycloendoperoxide intermediates.[8] However, this fascinating work was never carried beyond *in vitro* studies nor was it determined whether PG-like compounds could be formed in biological fluids containing unsaturated fatty acids.

Previously, we had shown that PGD_2 derived from the cyclooxygenase is primarily metabolized *in vivo* in humans to form 9α,11β-PGF_2 by the enzyme 11-ketoreductase.[11] In aqueous solutions, however, PGD_2 is an extremely unstable compound that undergoes isomerization of the lower side chain and these isomers can likewise be reduced by 11-ketoreductase to yield isomers of 9α,11β-PGF_2.[12] In the course of studies undertaken to further characterize these compounds utilizing a gas chromatographic (GC)/mass spectrometric (MS) assay, we found that in plasma samples from normal volunteers that were processed and analysed immediately, a series of peaks were detected possessing characteristics of F-ring PGs (Fig. 1). Surprisingly, however, when plasma samples that had been stored at − 20°C for several months were reanalysed, identical chromatographic peaks were detected but levels of putative PGF_2-like compounds were up to 100-fold higher.[6] Subsequently, studies led to the conclusion that these PGF_2-like compounds were generated in both freshly processed and stored plasma, not by a cyclooxygenase derived

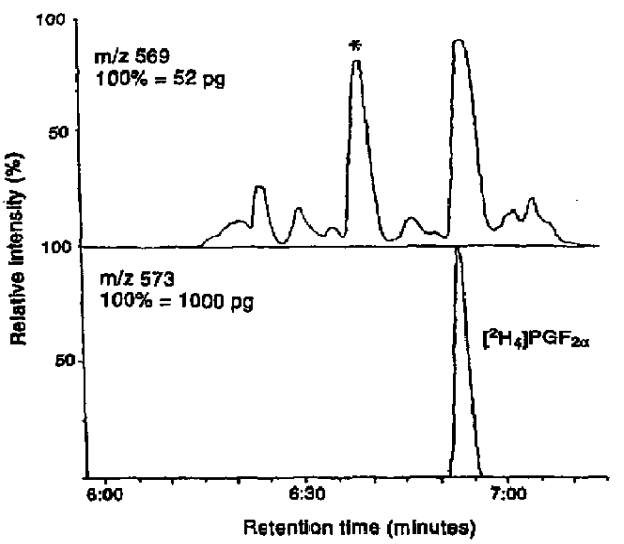


Fig. 1. Analysis of F_2-IsoPs in a plasma sample from a normal human volunteer. The m/z 569 ion current chromatogram represents endogenous F_2-IsoPs. The m/z 573 chromatogram represents $[^2H_4]PGF_{2\alpha}$ internal standard. The peak represented by the asterisk (*) is the one routinely quantified for F_2-IsoPs. The concentration of F_2-IsoPs in this plasma sample was 48 pg/ml.

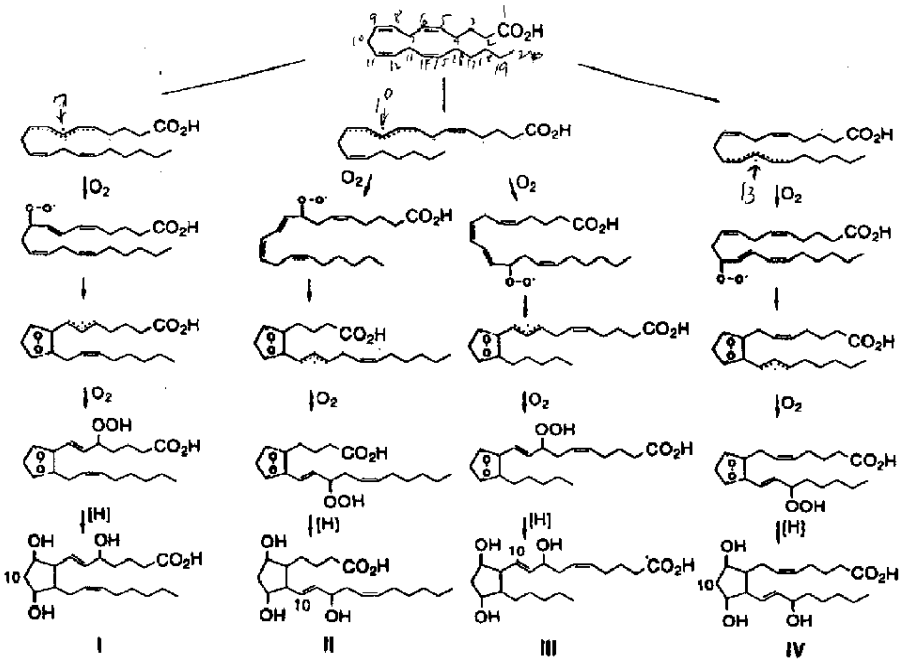


Fig. 2. Mechanism of formation of the F_2-IsoPs. This pathway leads to the formation of four regioisomers (I–IV). For simplicity, stereochemical orientation is not indicated. Each regioisomer theoretically comprises a mixture of eight racemic diastereomers. Reprinted with permission from Academic Press from *Analytical Biochemistry* **184**, 1–10 (1990).

mechanism, but non-enzymatically by autoxidation of plasma arachidonic acid.[6,13] Because these compounds contain F-type prostane rings, they will heretofore be referred to as F_2-isoprostanes (F_2-IsoPs).

III. MECHANISM OF FORMATION OF THE ISOPROSTANES

A mechanism to explain the formation of the F_2-IsoPs is outlined in Fig. 2 and is analogous to that proposed by Pryor and colleagues for the generation of bicycloendoperoxide intermediates resulting from the peroxidation of polyunsaturated fatty acids.[8] Precursor arachidonic acid shown at the top of the figure initially undergoes abstraction of an allylic hydrogen atom to yield an arachidonyl radical. Subsequently, there is insertion of oxygen to yield peroxyl radicals. Depending on the site of hydrogen abstraction and oxygen insertion, four different peroxyl radical isomers are formed. Endocyclization of the radicals then occurs, followed by the addition of another molecule of oxygen to yield four bicycloendoperoxide (PGG_2-like) regioisomers. These intermediates are then reduced to F_2-IsoPs. Each of the four regioisomers can theoretically comprise eight racemic diastereomers. Thus, a total of 64 different compounds can be generated by this process, although as discussed below, the formation of some is favored over others. Recently, in support of the proposed mechanism of formation, we have obtained direct evidence both *in vitro* and *in vivo* that each of the four classes of regioisomers are formed.[14] In addition, as might be expected, compounds comprising regioisomers I and IV predominate owing to the fact that regioisomers II and III derive from the same arachidonyl radical precursor.

Several structural aspects of the F_2-IsoPs are noteworthy in comparison with cyclooxygenase derived PGs. Since F-ring compounds derive from the reduction of endoperoxide intermediates, the hydroxyls on the prostane ring must be oriented *cis*, although they can be α,α or β,β.[6,10] In addition, unlike cyclooxygenase derived PGs, non-enzymatic generation of the IsoPs favors compounds in which the side chains are predominantly oriented *cis* in relation to the prostane ring.

IV. FORMATION OF ISOPROSTANES *IN VIVO*

As noted above, we initially discovered IsoPs as products of the oxidation of plasma arachidonic acid which had been stored at $-20°C$. Impressed at the facile formation of these compounds, we sought to determine if these compounds might also be formed *in vivo*. Several observations suggested that this might be the case. First, as mentioned, we were able to detect measurable quantities of F_2-IsoPs in fresh human plasma from normal volunteers analysed immediately at levels of 35 ± 6 pg/ml ($n = 12$).[13,15] However, since large quantities of IsoPs can be generated *ex vivo*, we were concerned whether these amounts represented true endogenous levels or whether they were formed *ex vivo* by autoxidation of plasma lipids. This latter possibility seemed unlikely for several reasons. First, plasma contains significant quantities of antioxidants and it has been reported that lipid peroxidation is inhibited until endogenous ascorbate is virtually entirely consumed.[16,17] Second, we found that drawing blood into syringes containing the antioxidant butylated hydroxytoluene (BHT) or the reducing substance triphenylphosphine, failed to reduce measured levels.[13,18] Third, we found that levels of F_2-IsoPs in urine from normal human volunteers were high (1.6 ± 0.6 ng/mg creatinine).[18] Urine contains only small amounts of lipids and thus it was unlikely that such substantial levels of these compounds would be generated *ex vivo*. Further support for this was the finding that urinary IsoP levels did not increase when urine was incubated at 37°C for up to 5 days.[13,18] Definitive evidence that IsoPs are, in fact, formed *in vivo* was demonstrated by showing that levels of compounds detected in the plasma of rats treated with either CCl_4 or the herbicide diquat to induce an oxidant injury were increased by up to 200 times levels measured in control rats.[13,19]

A second interesting aspect related to the formation of isoprostanes is that they are formed *in situ* esterified to phospholipids *in vivo*. It is known that only small amounts of arachidonic acid are present in the unesterified state and the vast majority exists esterified to phospholipids.[20] Thus, we explored the possibility that F_2-IsoPs are initially formed esterified to phospholipids and are subsequently released in the free form by phospholipases. This was important because it counters the accepted dogma that prostanoids do not exist esterified in phospholipids. In support of the hypothesis that IsoPs are initially formed *in situ* on phospholipids, we examined the time course of appearance of increases in levels of F_2-IsoPs esterified in liver phospholipids and free in the circulation following administration of CCl_4 to rats to induce an oxidant injury.[19] As shown in Fig. 3, levels of esterified IsoPs increased rapidly, reaching half maximum concentrations in the liver within 15 min, while the appearance of increases in the circulation was delayed significantly.[19] Direct evidence for the formation of F_2-IsoPs esterified to phospholipids was obtained when a lipid extract of liver tissue from rats treated with CCl_4 was subjected to HPLC purification using a straight phase system that separates phosphatidylcholine from

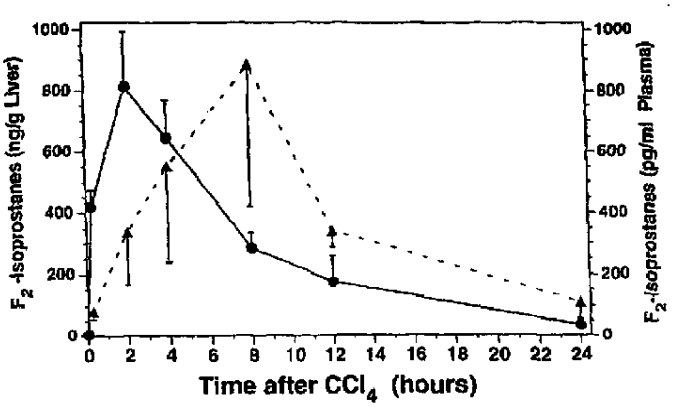


Fig. 3. Time course of the appearance of lipid-esterified F_2-IsoPs in liver (circles) and free compounds in the plasma (triangles) in rats after the orogastric administration of CCl_4 (1 ml/kg). Reprinted with permission from W. B. Saunders from *GI Clinics of North America* 25, 409–427 (1996).

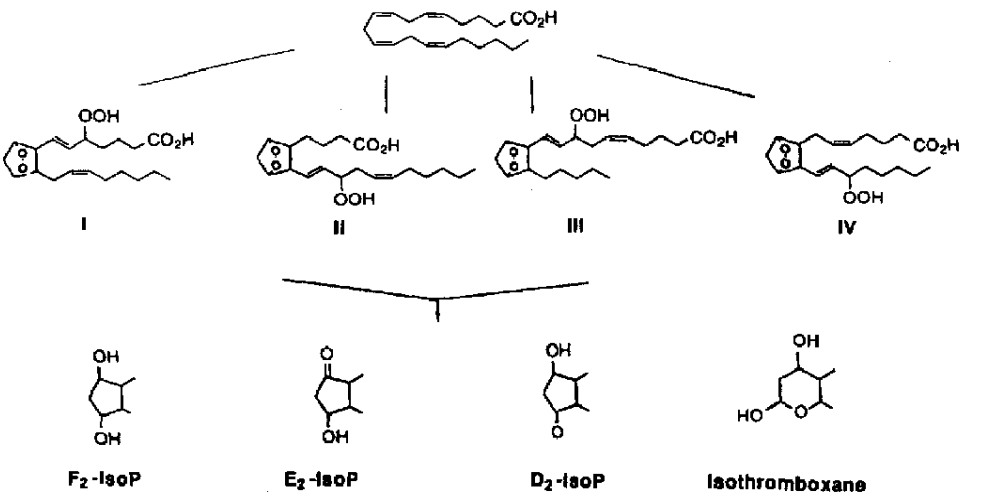


Fig. 4. Potential fates of the bicycloendoperoxide intermediates of the IsoPs (regioisomers I–IV) derived from the peroxidation of arachidonic acid. The endoperoxides undergo either reduction to F_2-IsoPs or rearrangement to D_2/E_2-IsoPs or isothromboxanes.

less polar lipids.[20] Fractions collected were then subjected to chemical hydrolysis and analysed for free F_2-IsoPs to detect those that contained esterified F_2-IsoPs. We found that fractions containing presumed esterified F_2-IsoPs eluted in a region that was more polar than unoxidized phosphatidylcholine.[20] Analysis of these fractions by fast atom bombardment MS definitely identified phosphatidylcholine species with palmitate or stearate esterified at the sn-1 postion and an F_2-IsoP at the sn-2 position. More detailed analysis of phospholipid-containing F_2-IsoPs have since been carried out utilizing collision induced dissociation tandem MS.[21]

As noted, after the administration of CCl_4 to rats, increased concentrations of F_2-IsoPs esterified in liver tissue can be detected followed by increased levels in the circulation.[19] This suggests that free compounds derive, at least in part, from the hydrolysis of IsoPs from phospholipids *in vivo*. It is reasonable to assume that the hydrolysis is catalysed by phopholipases. *In vitro*, we have found that bee (*Apis mellifera*) venom phospholipase A_2 efficiently hydrolyses IsoPs from lipids,[20] although the phospholipase(s) responsible for the hydrolysis of IsoPs *in vivo* remains to be established.

After determining that IsoPs are initially formed by peroxidation of arachidonic acid esterified to tissue lipids, we have analysed a variety of normal animal tissues for levels of esterified F_2-IsoPs, including liver, testes, heart, brain, skeletal muscle, aorta tissue, ocular lens, kidney, and lung and found detectable levels in all of these tissues.[18] Analysis of human tissues has been limited to gastric biopsies, where levels of F_2-IsoPs in the ng/g tissue are present. In addition, F_2-IsoPs are detectable in human cerebrospinal fluid at pg/ml concentrations and appear to be increased in certain settings of central nervous system inflammation (unpublished data). These findings are consistent with the fact that detectable levels of unesterified F_2-IsoPs are present in all normal biological fluids from both animals and humans that have been tested to date.

A. D_2/E_2-Isoprostanes

The cyclooxygenase derived endoperoxide intermediate, PGH_2, is unstable and rapidly rearranges in aqueous solutions to PGD_2 and PGE_2 with a half-life of several minutes.[22] Analogously, it was reasonable to suspect that the intermediate IsoP endoperoxides, if not efficiently reduced to F_2-IsoPs, may also rearrange to form D- and E-ring IsoPs (Fig. 4). Experiments were carried out to explore this possibility which convincingly demonstrated that D_2/E_2-IsoPs are also formed *in vivo*.[23] Levels of D_2/E_2-IsoPs esterified in a variety of

tissues of the rat were found to be approximately one-third to one-fourth the levels of F_2-IsoPs (Fig. 5). However, in contrast to F_2-IsoPs, D_2/E_2-IsoPs cannot be detected in the circulation of humans or rats under normal circumstances, although they can be detected in the circulation of rats that have been administered CCl_4 to induce severe lipid peroxidation. The reason for this is not clear but may be due to differences in the rate of the metabolic clearance of D_2/E_2- and F_2-IsoPs.

B. Isothromboxanes

It has previously been shown that the cyclooxygenase derived endoperoxide, PGH_2, can also rearrange non-enzymatically to form small quantities of thromboxane A_2, a process that can be catalysed by iron-containing porphyrin compounds.[24] Therefore, we examined whether the IsoP endoperoxides may also rearrange to form isothromboxane compounds *in vivo* (Fig. 4). Interestingly, we have recently provided convincing evidence that this does occur.[25] Using an assay we had previously developed for quantification of cyclooxygenase derived thromboxane B_2, we were able to detect significant amounts of isothromboxanes derived from the free radical catalysed peroxidation of arachidonic acid both *in vitro* and *in vivo*. Structural identification of these compounds was confirmed employing various chemical and mass spectrometric approaches. Levels of isothromboxanes esterified to tissue lipids, e.g. in liver, are similar to the levels of D_2/E_2-IsoPs (Fig. 5). Thus, these studies provide evidence for the formation of a third class of IsoPs produced via the free radical catalysed peroxidation of arachidonic acid. Studies are currently underway to determine whether other compounds might be formed via the IsoP pathway. In this regard, we have recently obtained intriguing evidence for the formation of levuglandin-like molecules (isolevuglandins) generated as a result of prostane ring opening of IsoP endoperoxide intermediates in a manner analogous to the formation of levuglandins from cyclooxygenase derived PGH_2.[26] These compounds are highly reactive keto-aldehyde containing moeities which rapidly adduct proteins and DNA *in vitro*. Thus, the formation of isolevuglandins and/or their adducts may participate in pathophysiological processes associated with oxidant injury.

C. Isoleukotrienes

In addition to IsoPs generated from unstable endoperoxide intermediates, other products derived from the free radical catalysed peroxidation of arachidonic acid have been recently characterized. Murphy and colleagues, employing mass spectrometric approaches, have reported that the *in vitro* oxidation of 1-hexadecanoyl-2-arachidonyl-glycerophosphocholine using Cu^{2+} and H_2O_2 results in the formation of a series of conjugated triene metabolites characterized as 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid compounds esterified to the glycerophosphocholine backbone.[27] Because of their structural similarity to leukotriene B_4, they have been named B_4-isoleukotrienes.

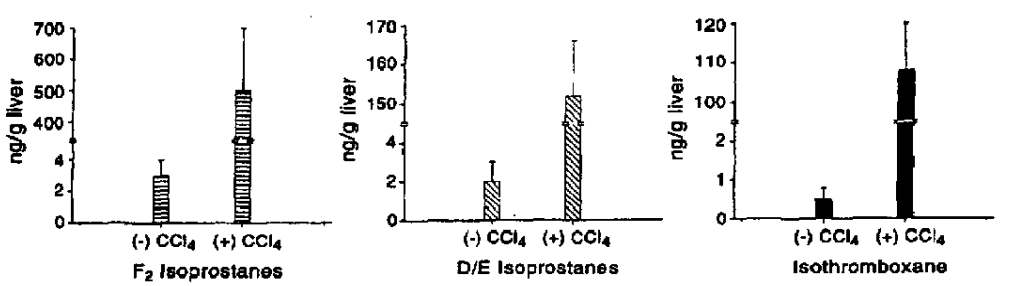
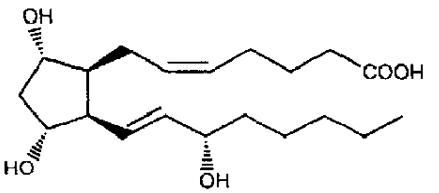


Fig. 5. Relative levels (ng/g liver) of different classes of IsoPs esterified in lipids from livers of rats at baseline or after administration of CCl_4 (1 ml/kg). Animals administered CCl_4 were sacrificed at 2 h.



8-iso-PGF$_{2\alpha}$ (15-F$_{2t}$-IsoP)

Fig. 6. Chemical structure of the IsoP, 8-iso-PGF$_{2\alpha}$ (15-F$_{2t}$-IsoP).

Interestingly, following their generation from arachidonylphosphatidylcholine and release by chemical saponification, several isoleukotrienes have been found to possess potent bioactivity. They increase intracellular calcium in neutrophils with an EC_{50} as low as 30 nM and this effect can be blocked with a leukotriene B_4 receptor antagonist. The identification of these molecules is important because it implies that the free radical catalysed oxidation of arachidonic acid not only results in the formation of IsoPs but also bioactive leukotriene-like compounds.

V. A NOMENCLATURE SYSTEM FOR THE ISOPROSTANES

Because multiple isoforms of F-ring and D/E-ring IsoPs can be formed from the free radical catalysed peroxidation of arachidonic acid, a need for systematic nomenclature has emerged. Thus, recently, we have proposed a facile nomenclature system that allows for the differentiation of the many isomeric IsoP compounds formed.[28] In addition, we are currently developing a nomenclature system for the isothromboxanes. The system for naming IsoPs follows standard rules of chemical nomenclature and conforms with PG convention. Briefly, as in this review, the term isoprostanes is abbreviated as IsoP. The initial reference to different classes of IsoPs as F_2-, E_2-, and D_2- is also utilized. Originally, the IsoPs were divided into four classes (I–IV) to refer to each of the different regioisomers (Fig. 2). We now name these four classes by the location of the side chain hydroxyl group, with C-1 being the carboxyl group consistent with standard PG nomenclature. Thus regioisomer I is a 5-F_2-IsoP, II is a 8-F_2-IsoP, III is a 12 F_2-IsoP, and IV is a 15-F_2-IsoP. The analogous nomenclature for the other IsoP classes is 5-E_2-IsoP, 5-D_2-IsoP, etc. The default absolute configuration of the side chain hydroxyl is (*S*). If the side chain hydroxyl is (*R*), the compound is denoted as "epi".

Additional structural features involved in compound nomenclature include the absolute configuration of the ring hydroxyls, which based on the mechanism of formation of IsoPs is only oriented *cis* although they can be either up or down. If the hydroxyls are oriented down (α), this is the default configuration. If they are oriented up (β) the compound is designated "ent". Further, the favored side chain configuration of IsoPs is *cis* relative to the prostane ring. Again, this is a default configuration while if the side chains are *trans*, the compound is designated "epi". Finally, compounds are then delineated in relation to whether the side chains are oriented *trans* or *cis* with respect to the cyclopentane ring hydroxyls and the notation of either "t" or "c" is placed as a subscript.

One of the F_2-IsoPs that has been studied extensively is 8-iso-PGF$_{2\alpha}$ (Fig. 6). Much of the interest in this compound derives from the fact it possesses potent bioactivity.[13] Although as discussed below, trivial amounts of this compound may be formed via the cyclooxygenase, free radical mediated non-enzymatic oxidation of arachidonic acid accounts for virtually all of the formation of this compound *in vivo*.[13] Thus, reference to this compound as a PG misses the distinction between cyclooxygenase derived PGs and IsoPs. In accordance with the proposed nomenclature, this compound should be designated 15-F_{2t}-IsoP.

To familiarize the reader with this new nomenclature, specific IsoPs discussed in the remainder of this article will be identified as both a PG and IsoP.

VI. IMPORTANCE OF THE DISCOVERY OF ISOPROSTANES

A. Analytical Ramifications

The discovery of IsoPs is important for several reasons. First, the finding that F_2-IsoPs can be generated in biological fluids *in vitro* has potentially important analytical ramifications for the analysis of prostanoids.[6] This applies to both physical and immunological methods of analysis. Particular precautions must be taken to avoid generation of IsoPs in lipid-containing biological fluids prior to analysis (see below). These compounds have similar chromatographic properties on thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas chromatography (GC) as $PGF_{2\alpha}$ and thus can confound an interpretation of whether a PGF_2 compound measured by physical methods, e.g. GC/MS, is enzymatically or non-enzymatically generated.[6] Further, antibodies used in immunoassays for cyclooxygenase derived PGF_2 compounds may potentially cross-react with F_2-IsoPs. For example, we have found that an antibody obtained commercially (Amersham Life Science) to the PGD_2 metabolite, $9\alpha,11\beta$-PGF_2, exhibits significant cross-reactivity with the complex mixture of F_2-IsoPs, even though the prostane ring hydroxyls in F_2-IsoPs are oriented *cis*.[6]

B. The Isoprostanes as an Index of Endogenous Lipid Peroxidation

A second important aspect of the discovery of IsoPs relates to the use of measurement of IsoPs as an index of lipid peroxidation or oxidant stress *in vivo*. It has been previously recognized that one of the greatest needs in the field of free radical research is the availability of a reliable non-invasive method to assess oxidative stress status *in vivo* in humans. This is the case because most methods available to assess oxidant stress *in vivo* previously have suffered from a lack of specificity and/or sensitivity or are unreliable.[5] However, a substantial body of evidence has been obtained that indicates measurement of IsoPs, i.e., in urine or plasma, provides a reliable non-invasive approach to assess lipid peroxidation *in vivo* and, as such, represents what appears to be a major advance in our ability to assess oxidative stress status in humans (see below). Further, the sensitivity of the mass spectrometric method of analysis appears sufficient to quantify levels of F_2-IsoPs in small biopsies of human tissue, e.g. gastic biopsies (unpublished data), which should permit an assessment of oxidant injury in key tissues of interest.

The ability to quantify F_2-IsoPs, therefore, will potentially allow for exploration of the role of free radicals in the pathophysiology of a wide range of human diseases. It also provides an extremely valuable tool to define the clinical pharmacology of antioxidant agents. There are trials either planned or underway examining the effect of antioxidants such as vitamin C, vitamin E, or others to prevent or ameliorate some of the pathology of diseases in which free radicals have been implicated. However, such studies are hampered by insufficient information regarding what doses and combinations of antioxidants are maximally effective. Measurement of IsoPs should provide a valuable approach to define the clinical pharmacology of antioxidants. In this respect, we have previously shown that the formation of F_2-IsoPs increases significantly in animals deficient in vitamin E and/or selenium.[13,29] In addition, administration of antioxidants has been shown to inhibit the formation of IsoPs in animal models of oxidant injury.[30] More recently, we have found that the administration of a combination of antioxidants at high doses (4 g/day vitamin C, 3200 IU/day vitamin E, and 300 mg/day β-carotene) to normal volunteers for a period of 2 weeks inhibited the formation of F_2-IsoPs esterified to plasma lipids by a mean 37% (unpublished data). Further, we have also examined the effect of administering 400 and 800 IU/day of vitamin E (α-tocopherol) for 2 weeks on plasma concentrations of unesterified F_2-IsoPs. In the group given 400 IU/day vitamin E, circulating concentrations of F_2-IsoPs decreased 25% while in the group taking 800 IU/day of vitamin E, plasma levels of F_2-IsoPs fell by 37%. The lower dose approached statistical significance while the higher dose produced a highly significant decrease in IsoP formation ($p = 0.007$) (unpublished data). These data suggest that measurement of IsoPs can be used

to quantitatively define the effects of antioxidants to inhibit free radical processes *in vivo* in humans. Such information would be important to long-term studies aimed at exploring the efficacy of antioxidants to prevent free radical mediated pathological tissue changes associated with particular diseases.

Levels of IsoPs in normal human plasma and urine exceed levels of cyclooxygenase derived PGs and thromboxane by more than an order of magnitude suggesting that the formation of IsoPs is a major pathway of arachidonic acid disposition.[13] Further, it is important to consider the relevance of the finding that levels of F_2-IsoPs are sufficient to be detected in every normal biological fluid that has been assayed; these include plasma, urine, cerebrospinal fluid, bile, and gastric juice. Previously, using other methods to assess lipid peroxidation, there had been little definitive evidence indicating lipid peroxidation occurs *in vivo* except under abnormal circumstances of marked oxidative stress. However, the finding of detectable levels of F_2-IsoPs in all normal animal and human biological fluids and esterified in normal animal tissues indicates that there is ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses, even in normal individuals. This finding may lend some support to the hypothesis that the normal aging process is due to enhanced oxidant damage of important biological molecules over time.[4] In this regard, it has been reported that there is a trend for the formation of F_2-IsoPs to increase with age in humans.[31]

C. *The Isoprostanes as Mediators of Oxidant Stress*

Another important aspect of the discovery of IsoPs, as discussed below, has been the finding that at least two IsoPs, 8-iso-PGE_2 (15-E_{2t}-IsoP) and 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP), exert potent biological activity.[13,23] Thus, these compounds may not simply be markers of lipid peroxidation, but may also participate as mediators of oxidant injury. The possibility that additional IsoPs will also be found to possess biological activity awaits the availability of additional synthetic compounds. The chemical synthesis of additional IsoPs is currently being undertaken by at least three laboratories and, recently, two additional compounds have become available (see below).[32] Regarding the biological activity of isothromboxanes, one might anticipate that, analogous to cyclooxygenase derived thromboxane, only the compounds with a thromboxane A_2-like ring, but not a thromboxane B_2-like ring structure, would be bioactive. Unfortunately, because of the marked instability of the thromboxane A_2 ring, it will be difficult, if not impossible, to synthesize isothromboxane A_2 compounds for purposes of biological testing.

VII. METHOD OF ANALYSIS OF THE ISOPROSTANES

The method that we have utilized for measurement of F_2-IsoPs is a GC/electron capture/negative chemical ionization MS assay.[13,18] It is highly sensitive with a lower limit of detection in the low picogram range. Further, it is highly accurate (precision = $\pm$ 6%, accuracy = 96%). Previously we have used either [$^2H_7$]9α,11β-PGF_2 synthesized in our laboratory or commercially available [$^2H_4$] $PGF_{2\alpha}$ as an internal standard, but recently [$^2H_4$] 8-iso-$PGF_{2\alpha}$, one of the more abundant F_2-IsoPs produced *in vivo*,[33] has become available commercially. Measurement of esterified levels of F_2-IsoPs in tissues is accomplished by measurement of free compounds following alkaline hydrolysis of a lipid extract of tissue.[18] IsoPs are analysed following conversion to pentafluorobenzyl ester trimethylsilylether derivatives. For quantification purposes, we quantify the (*) peak shown in the m/z 569 chromatogram in Fig. 1. We have previously shown that 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) comprises a significant proportion of the F_2-IsoPs represented by this chromatographic peak.[33]

While highly accurate, the mass spectrometric method of assay is labor intensive and the technology is not widely available. However, both commercial enterprises and academic investigators have developed or are developing immunoassays for specific F_2-IsoPs,[34] which should expand research in this area. Currently, three immunoassay kits

are commercially available. Further, a method was recently reported for measurement of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) using an immunoaffinity column for purification coupled with quantitation by mass spectrometry.[35]

A. Precautions Associated with Isoprostane Measurements

IsoPs can be readily generated *in vitro* from the autoxidation of arachidonic acid in biological fluids.[6] Thus, precautions must be taken to prevent artifactual generation of IsoPs by autoxidation during storage and sample processing. Although we reported that IsoPs are generated by autoxidation in lipid-containing samples stored at $-20°C$, we have found that autoxidation does not occur in lipid containing samples, e.g. plasma, that are initially snap frozen in liquid nitrogen and stored at $-70°C$ for up to 6 months.[18] However, once thawed, samples should be assayed immediately and not refrozen. Tissue samples collected for measurement of esterified IsoPs also must either be analysed immediately or snap frozen in liquid nitrogen and stored at $-70°C$. Autoxidation is not a problem with urine samples owing to the fact that urine contains only small quantities of lipid; we have found that levels of F_2-IsoPs do not increase, even when urine is incubated at 37°C for 5 days.[18]

In addition to precautions necessary during storage, precautions are also required during processing of certain types of samples for quantification of IsoPs, specifically addition of antioxidants. Measurement of free unesterified levels of IsoPs in biological fluids, even those that contain lipid, does not require the addition of antioxidants. However, autoxidation is of primary concern in the analysis of esterified IsoPs in tissues and, particularly, plasma lipoproteins.[18] This may be due to the more extensive manipulation required for analysis of esterified IsoPs. These samples first require a Folch lipid extraction followed by evaporation under N_2 of a large volume of organic solvent. Then the samples must be subjected either to base hydrolysis, in the case of F_2-IsoPs, or enzymatic liberation of D_2/E_2-IsoPs with bee venom phospholipase A_2, after which free compounds are extracted using a C-18 cartridge.[18,23] We have found that the addition of 0.005% butylated hydroxytoluene (BHT) to the organic phase during the Folch lipid extract prevents artifactual generation of IsoPs by autoxidation in tissue samples.[18] Autoxidation occurs more readily, however, when IsoPs esterified to plasma lipoproteins are measured. In this case, addition of BHT does not fully prevent autoxidation. However, if the reducing agent, triphenylphospine (0.5%) is also added to the organic phase along with BHT, autoxidation during processing of plasma lipoproteins can be effectively suppressed.[15,18]

An attractive approach for assessing total endogenous F_2-IsoP production is measurement of F_2-IsoP metabolites.[36] Measurement of metabolites of F_2-IsoPs in urine has the advantage not only of circumventing the problem of artifactual generation of F_2-IsoPs *ex vivo* by autoxidation but can also provide an integrated assessment of F_2-IsoP production over time. It has been shown that unmetabolized cyclooxygenase derived PGs in urine derive largely from local formation in the kidney.[37,38] Although currently we do not know whether urinary unmetabolized F_2-IsoPs derive from the kidney, it is likely that, at least in part, this is the case. In this respect, we found that there was a very high correlation between the urinary excretion of F_2-IsoP metabolites and circulating concentrations of F_2-IsoPs under circumstances associated with mild/moderate overproduction of F_2-IsoPs in humans who were heavy smokers ($r = 0.97$, $p < 0.001$).[15] In contrast, there was a poor correlation between the urinary excretion of unmetabolized F_2-IsoPs and plasma levels of F_2-IsoPs ($r = 0.29$, $p = 0.41$) (unpublished data). This study suggests that although the levels of unmetabolized F_2-IsoPs in urine may increase when the production of F_2-IsoPs from extrarenal sources is enhanced due to filtration of F_2-IsoPs from the circulation, measurement of unmetabolized urinary F_2-IsoPs may not be as sensitive to modest increases in systemic production of IsoPs as measurement of circulating concentrations of F_2-IsoPs or urinary F_2-IsoP metabolites.

VIII. ISOPROSTANE METABOLISM

Knowledge of the metabolic fate of IsoPs is limited. We previously carried out experiments in the rat exploring the time course of disappearance of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) from the circulation. From the data obtained, it was determined that the $t1/2$ of the clearance of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) from the circulation was $\sim$ 16 min.[19] Although not examined directly, it is likely, analogous to the metabolism of other prostanoids, that the lung is a major site of metabolic clearance of F_2-IsoPs from the circulation.[39] This is supported by the finding that the creation of a porta-caval shunt and ligation of the hepatic artery in rats, completely eliminating clearance of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) by the liver, only prolonged the $t1/2$ of the clearance of 8-iso-$PGF_{2\alpha}$ from the circulation by 5 min, to 21 min.[19]

Recently, Schuster and colleagues have characterized an inward prostaglandin transporter (PGT) expressed in high levels in various rat tissues, most notably the lung.[40] This transporter is encoded by rat matrin F/G gene DNA and has a 37% homology with the rat liver bromosulfophthalein-bile salt transporter (oatp). It possesses high affinity for various PGs, most notably PGE_2 and $PGF_{2\alpha}$. Interestingly, we recently found that the isoprostanes 8-iso-PGE_2 (15-E_{2t}-IsoP) and 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) are also readily transported by PGT.[41] These observations suggest that the IsoPs are rapidly taken up and presumably metabolized by tissues in a manner similar to cyclooxygenase derived PGs.

Within the past year, we have explored the metabolic fate of 8-iso-$PGF_{2\alpha}$(15-F_{2t}-IsoP) in humans using radiolabeled 8-iso-$PGF_{2\alpha}$.[42] Surprisingly, 43% of excreted radioactivity would not extract into ethyl acetate, suggesting the presence of very polar material, perhaps polar conjugates.[43] We identified the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) as 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP). This metabolite represented 29% of the total extractable recovered radioactivity in urine. It is interesting that the $\triangle^5$ double bond in this metabolite had been reduced since this reduction is not a prominent feature of the metabolism of cyclooxygenase derived eicosanoids.[39] The reason for this is unclear, although perhaps the inversion of the stereochemistry of the upper side chain renders 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) or 2,3-dinor-8-iso-$PGF_{2\alpha}$ (2,3-dinor-15-F_{2t}-IsoP) a better substrate for the reductase(s) which reduce the $\triangle^5$ double bond. The importance of the identification of the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) is that it provides the basis for development of an assay for this compound as a means to obtain an integrated assessment of total endogenous F_2-IsoP production in humans.

IX. BIOLOGICAL ACTIVITY OF THE ISOPROSTANES

Because IsoPs are isomeric to bioactive cyclooxygenase derived PGs, we examined whether IsoPs may not be simply markers of lipid peroxidation but also possess biological activity, in which case they may participate as mediators of oxidant injury. As stated, in contrast with cyclooxygenase-derived PGs, the side chains of IsoPs are predominantly oriented *cis* in relation to the prostane ring.[6] Thus, one of the compounds that would be predicted to be formed would be 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP). Using high resolving HPLC approaches for purification of individual F_2-IsoPs from the complex mixture using radiolabeled 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) as a marker, we found that 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) is, in fact, one of the more abundant F_2-IsoPs that is produced *in vivo*.[33] This compound was synthesized and made available to us in 1990 by Gordon Bundy (Upjohn) and is now commercially available. Because the E-ring IsoPs are formed by chemical rearrangement of the IsoP endoperoxides, 8-iso-PGE_2 (15-E_{2t}-IsoP) would also be expected to be formed from 8-iso-PGG_2 (15-G_{2t}-IsoP).[23] This compound has recently become available commercially also.

Because urinary F_2-IsoP levels are high and may in part derive from local production in the kidney, we initially examined whether 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) exerted any biological effects on renal function in the rat. Interestingly, it was an extremely potent renal vasoconstrictor, reducing glomerular filtration rate and renal blood flow by 40–45% in the

low nanomolar range.[13,44] During systemic infusion of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) that was associated with a fall in renal blood flow of ~50%, no alteration in systemic blood pressure occurred, suggesting a selective effect of this IsoP on renal vasculature. The primary action of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) in the glomerulus is constriction of the afferent renal arteriole, leading to a drop in glomerular capillary pressure.[44,45] 8-Iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) has also been found to be a potent pulmonary artery vasoconstrictor in rabbits and rats and to cause bronchoconstriction in the rat lung.[46,47] Further, 8-iso-$PGF_{2\alpha}$ (15-F_{2t} -IsoP) has also been shown to induce mitogenesis in vascular smooth muscle cells and induce endothelin-1 release from bovine aortic endothelial cells.[44,45,48] Surprisingly, the E-ring IsoP, 8-iso-PGE_2 (15-E_{2t}-IsoP), also is a potent renal vasoconstrictor, approximately equipotent with that of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP).[23] This was an unexpected finding because in most systems, cyclooxygenase derived PGE_2 and $PGF_{2\alpha}$ have opposing biological effects, which has been attributed to the differences in ring structure. In particular, PGE_2 is a vasodilator whereas $PGF_{2\alpha}$ is a vasoconstrictor.[49] The finding that 8-iso-PGE_2 (15-E_{2t}-IsoP) and 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) are both potent vasoconstrictors in the renal vascular bed suggests that the stereochemistry of the side chains, rather than ring structure, may be an important determinant of the biological actions of IsoPs.

More recently, two other IsoPs have been chemically synthesized. They are 15*R*-8-iso-$PGF_{2\alpha}$ (15-epi-15-F_{2t}-IsoP) and a 5-F_2-IsoP [Regioisomer I (Fig. 2)], 5-F_{2t}-IsoP.[32] We have examined, in a preliminary manner, the biological activity of the former compound on platelet function. Unlike 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP), however, 15R-8-iso-$PGF_{2\alpha}$ (15-epi-15-F_{2t}-IsoP) has no effect on platelet aggregation. These results suggest that the (*S*) orientation of the side chain hydroxyl may be necessary for this biological activity.

A. Evidence for a Unique Isoprostane Receptor

A series of investigations examining the mechanism by which 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) and 8-iso-PGE_2 (15-E_{2t}-IsoP) exert their biological actions on vascular smooth muscle have led to unexpected findings suggesting that these IsoPs exert their effects by interacting with a unique receptor. We had initially found that the renal vasoconstricting actions of these compounds could be abrogated by the thromboxane receptor antagonist SQ29548, suggesting that these compounds interacted with thromboxane receptors.[44] Interestingly, however, when incubated with platelets, 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) (10^{-6} and 10^{-5} M) caused a slight shape change and only at very high concentrations (10^{-4} M) induced reversible but not irreversible aggregation.[50] 8-iso-PGE_2 (15-E_{2t}-IsoP) caused a modest degree of irreversible aggregation of platelets from a few individuals at concentrations of 10^{-5} and 10^{-4} M but in most, it only caused reversible aggregation at these concentrations.[51] In contrast, both IsoPs are more potent as antagonists of thromboxane receptor agonist-induced platelet aggregation. That these compounds act primarily as antagonists of the thromboxane receptor in platelets would not be consistent with their interacting with thromboxane receptors unless the platelet and vascular smooth muscle thromboxane receptors were different. This is unlikely, however, since only a single thromboxane receptor gene has been identified,[52] although splicing variants of the thromboxane receptor have been characterized which appear to have similar ligand binding characteristics and phospholipase C activation but oppositely regulate adenylyl cyclase activity.[53,54] Nonetheless, 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) probably interacts with both receptor isoforms.[55]

A second explanation for the above findings would be that the IsoPs interact with a unique receptor on vascular smooth muscle distinct from the thromboxane receptor. In order to explain the fact that the vascular effects of these compounds are abrogated by SQ29548, one could argue that this putative "IsoP" receptor is structurally similar to the thromboxane receptor. Suggesting the presence of a receptor for IsoPs distinct from the thromboxane receptor was the finding that, whereas the IsoPs were more potent in inducing a functional response on vascular smooth muscle cells than thromboxane receptor agonists, they were much weaker than thromboxane receptor agonists in displacing

thromboxane receptor ligand binding.[45] Preliminary radiolabeled binding studies are also consistent with the presence of a unique receptor for these IsoPs.[56] In these experiments, low affinity and high affininty binding sites were found for 8-iso-$PGF_{2\alpha}$ both in vascular smooth muscle cells and endothelial cells. It was speculated that the former may represent binding to the thromboxane receptor and the latter binding to an "IsoP receptor". Although these data suggest a novel "IsoP receptor", definitive proof must await the results of molecular approaches aimed at cloning this receptor.

X. QUANTIFICATION OF ISOPROSTANES AS AN INDEX OF OXIDANT STRESS

A. Overview

As mentioned, a number of methods developed to detect free radical injury have been found to be unreliable, particularly when applied to *in vivo* situations. However, a large number of studies suggest that measurement of IsoPs represents an important advance for assessing oxidative stress status.[57] Prior to discussing these studies, however, it is important to point out that IsoPs are specific products of lipid peroxidation resulting from the free radical catalysed peroxidation of arachidonic acid. In this regard, it had been reported that small quantities of the F_2-IsoP, 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP), can be produced as a byproduct of the cyclooxygenase enzyme.[58] Further, it was recently demonstrated that very small quantities of 8-iso-$PGF_{2\alpha}$ are formed by PGH synthase-1 during aggregation of human platelets *in vitro* and by PGH synthase-2 in human monocytes.[59,60] However, the amounts of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) formed by activated platelets and monocytes are no greater than 1/100th of the amount of thromboxane formed. In addition, we originally demonstrated that the administration of high doses of cyclooxygenase inhibitors to normal humans does not suppress the intensity of the peak in which 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) elutes when analysed by GC/MS.[13] This has been confirmed by others[61] and indicates that the relative contribution of enzymatic generation of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) *in vivo* is inconsequential compared with the amounts formed non-enzymatically. However, it was speculated that in settings of enhanced platelet aggregation *in vivo*, the contribution of the cyclooxygenase to levels of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}IsoP) might be significant. However, results of a recent study do seem to bear out this concern.[62] Further, we have measured plasma levels of F_2-IsoPs by quantifying the intensity of the peak in which 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) elutes from the GC column in a patient with unusually severe systemic mastocytosis associated with a dramatic overproduction of PGD_2 ($\sim$ 100-fold above normal). In this patient, the level measured was in the normal range (unpublished data). Thus, even in pathologic situations associated with extreme increases in cyclooxygenase activity, enzymatic generation of 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) remains insignificant in relation to the amounts formed non-enzymatically *in vivo*. In addition, simply measuring one or more of the other F_2-IsoPs can provide a specific and reliable marker of free radical catalysed lipid peroxidation because 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) is the only F_2-IsoP that has been shown as capable of being produced by an enzymatic process.[58]

B. In Vitro *Studies*

A number of studies have been carried out involving the quantification of F_2-IsoPs in *in vitro* systems of lipid peroxidation, and F_2-IsoP formation has been compared with other markers of lipid peroxidation. This work has demonstrated the utility of measuring these compounds as a reliable index of lipid peroxidation *in vitro*.

The formation of F_2-IsoP has been compared with malondialdehyde (MDA) in Fe/ADP/ascorbate induced peroxidation of rat liver microsomes.[63] MDA is one of the most commonly used measures of lipid peroxidation and was quantified in these studies by measuring thiobarbituric acid reacting substances. Both F_2-IsoP and MDA formation increased in parallel in a time dependent manner and correlated with the loss of arachidonic acid and with increasing oxygen concentrations up to 21%. Although the

formation of F_2-IsoP correlated with other measures of lipid peroxidation in this *in vitro* model, as discussed below, measurement of F_2-IsoPs is superior to measurements of MDA as an index of lipid peroxidation *in vivo*.

We and others have also carried out studies examining the formation of F_2-IsoP in low density lipoproteins (LDL) exposed to various oxidizing conditions *in vitro*. Much of the interest in examining this stems from the hypothesis that oxidation of LDL *in vivo* converts it to an atherogenic form which is taken up by macrophages in the vessel wall. Subsequent activation of these cells may play an important role in the development and progression of atherosclerotic lesions in humans.[64] Thus, we have performed studies examining the formation of F_2-IsoP in LDL that is oxidized to determine whether measurement of F_2-IsoP esterified to lipoproteins may provide an approach to assess lipoprotein oxidation *in vivo*.[16] These studies are also of interest because the F_2-IsoP, 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) is a vasoconstrictor and induces mitogenesis in vascular smooth muscle cells[13,44] and these effects may be of relevance to the pathophysiology associated with atherosclerosis. In these studies, either plasma lipids or purified LDL from humans were peroxidized with Cu^{2+} or the water soluble oxidizing agent 2,2-azo-bis-2-amidinopropane (AAPH).[16] The formation of F_2-IsoPs was compared with other markers of lipid peroxidation including formation of cholesterol ester hydroperoxides, phospholipid hydroperoxides, loss of antioxidants and changes in the electrophoretic mobility of LDL. In plasma oxidized with AAPH, increases in the formation of F_2-IsoP paralleled increases in lipid hydroperoxide formation and occurred only after depletion of the antioxidants ascorbate and ubiquinol-10. In purified LDL that was oxidized, formation of F_2-IsoP again correlated with increases in lipid hydroperoxides and increases in the electrophoretic mobility of LDL. Further, increased F_2-IsoP formation occurred only after depletion of the antioxidants α-tocopherol and ubiquinol-10. Similar findings have been reported by Gopaul and colleagues when LDL is oxidized in the presence of endothelial cells or Cu^{2+}.[65] More recently, Fitzgerald and colleagues have reported large increases in 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) in LDL oxidized *in vitro* in the presence of macrophages stimulated with zymosan.[55] The reason for this enhanced formation of IsoP is probably due to activation of superoxide production.

There has been significant interest in the role that the macrophage 15-lipoxygenase enzyme might play in the oxidation of lipoproteins in the vascular wall and the relation to atherosclerosis.[66] In support of a role for this enzyme in the oxidation of LDL *in vivo*, it was recently shown that 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) formation in LDL incubated with stimulated macrophages isolated from mice genetically engineered with a targeted disruption of the 15-lipoxygenase gene was significantly less than when LDL was incubated with macrophages isolated from control animals.[67]

There has also been interest in the potential role of the oxidant peroxynitrite in LDL oxidation. Peroxynitrite is the coupling product of nitric oxide and superoxide. We examined the formation of F_2-IsoPs in LDL exposed to peroxynitrite and found that peroxynitrite catalyses the formation of F_2-IsoPs in a concentration dependent fashion, which correlated with increases in the electrophoretic mobility of LDL.[68]

Taken together, these studies suggest that quantification of F_2-IsoP esterified to lipoproteins may provide a useful approach to assessing oxidation of LDL *in vivo*. Supporting this notion is our recent finding that levels of isoprostanes esterified to plasma lipids are increased in smokers (see below).

C. F_2-Isoprostane Quantification in Animal Models of Oxidant Stress

Evidence that measurement of IsoPs provides a valuable approach to assess oxidative stress status *in vivo* emerged from early studies that we carried out related to the discovery of these compounds.[13,19] Importantly, as mentioned previously, we can detect measurable levels of IsoPs in virtually every animal and human biological fluid and tissues that have been analysed. This allows the definition of a normal range and even small increases in IsoP formation can be quantified. Further, overproduction of IsoPs has been well

documented to occur in settings of oxidant injury. Initial work *in vivo* with the IsoPs employed two models of liver injury in rats in which lipid peroxidation had been implicated as an important factor: administration of CCl_4 to normal rats and diquat to selenium (Se) deficient rats.

1. *CCl_4-Induced Lipid Peroxidation*

Administration of hepatotoxic doses of CCl_4 to rats caused hepatic lipid-esterified IsoPs to increase 200-fold within 1 h with a subsequent decline over 24 h (Fig. 3).[19,69] Plasma-free and lipid-esterifed IsoP concentrations increased after liver levels and peaked at 4–8 h after CCl_4 administration (Fig. 3).[19] Elevated IsoP levels were also documented in the bile.[70] Increased formation of F_2-IsoPs is proportional to CCl_4 dose administered.[69] Further, animals administered agents such as isoniazid or phenobarbital which induce hepatic cytochrome P-450 enzymes and increase CCl_4 metabolism have IsoP levels higher than animals administered only CCl_4.[19] In addition, depletion of endogenous glutathione stores markedly increases F_2-IsoP levels after the administration of CCl_4.[19] On the other hand, circulating and tissue levels of F_2-IsoP can be decreased compared with animals administered CCl_4 alone by pretreatment of rats with the antioxidant lazaroid U78517 or cytochrome P-450 inhibitors such as 4-methylpyrazole or SKF525A.[19,30]

Studies carried out utilizing CCl_4 to induce oxidant injury in the rat have also illustrated that quantification of F_2-IsoP provides a much more sensitive and accurate method to assess lipid peroxidation *in vivo* compared with other markers. As an example, following administration of CCl_4 to rats, levels of F_2-IsoPs esterifed to lipids increased greater than 80-fold, whereas levels of MDA in the liver increased only 2.7-fold.[63] In another study, we also found that measuring F_2-IsoP afforded a more sensitive indicator of CCl_4-induced lipid peroxidation compared with measurement of lipid hydroperoxides by mass spectrometry.[30]

2. *Diquat-induced Hepatic and Renal Toxicity*

Diquat is a dipyridyl herbicide that undergoes redox cycling *in vivo* generating large amounts of the superoxide anion. This compound causes hepatic and renal injury in rats and this effect is markedly augmented in animals deficient in Se, a trace element that is required for the enzymatic activities of glutathione peroxidase and other antioxidant proteins.[71] Previous studies have suggested that lipid peroxidation might be involved in the tissue damage associated with this agent. To study whether F_2-IsoPs were generated in increased amounts in association with diquat administration to Se-deficient animals, levels of F_2-IsoPs were quantified in plasma and tissues from Se-deficient rats following diquat administration. Se-deficient rats administered diquat showed 10- to 200-fold increases in plasma F_2-IsoPs and the sources of the IsoPs were determined primarily to be the kidney and liver.[69] Additional studies have also shown that GSH depletion increases IsoP levels significantly after the administration of diquat to rats.[72]

3. *Nutritional Antioxidant Deficiency*

We have carried out a large number of studies examining the role of the antioxidant micronutrients, vitamin E and Se, in IsoP formation. Rats raised on a diet deficient in both Se and vitamin E from weaning begin to lose weight and frequently die of massive hepatic necrosis.[73] *In vitro* studies demonstrating that vitamin E blocks production of lipid peroxidation suggested that uncontrolled lipid peroxidation might be responsible for the liver injury seen in vitamin E/Se-deficient animals although clear-cut data supporting this hypothesis were scarce.[74] In an effort to examine the role of oxidant injury in combined vitamin E/Se deficiency, we quantified F_2-IsoP in plasma and tissues of deficient rats without any exogenous oxidant stress. Interestingly, plasma F_2-IsoPs in rats raised on a doubly deficient diet were 6-fold higher than in rats raised on a control diet.[29] In addition, there were significant increases in phospholipid esterified F_2-IsoP levels in the tissues of

deficient animals including the liver, lung, kidney, heart and skeletal muscle. During this study, several animals maintained on a deficient diet developed spontaneous fulminant hepatic necrosis. Plasma and tissue IsoPs quantified in these animals just before death showed massive increases in IsoP levels, up to several hundred-fold, supporting the contention that lipid peroxidation is increased in animals deficient in both vitamin E and Se.[69]

In additional studies, we have also found markedly increased baseline levels of isoprostanes both in plasma and tissues of animals deficient in vitamin E alone.[29] On the other hand, animals deficient in Se alone do not have significantly increased F_2-IsoP levels in tissues or plasma compared with Se-replete animals unless they are exposed to an oxidant stress.[29]

4. *F_2-Isoprostane Levels in other Animal Models of Oxidant Injury*

A role for free radicals and lipid peroxidation in alcoholic liver damage has been controversial for many years. Recently, Nanji and colleagues reported increased plasma and lipid isoprostanes in rats fed ethanol chronically.[75] In a separate study, cimetidine given to rats to inhibit ethanol metabolism prevented the increase in F_2-IsoP formation and also prevented ethanol-induced liver injury.[76]

In rats rendered Cu-deficient by reduction of dietary Cu, Cu/Zn superoxide dismutase activity is markedly reduced. In these animals, we found significantly increased levels of F_2-IsoPs esterified in plasma lipoproteins (mean 2.5-fold increased) compared with normal control animals (unpublished data). In addition, there was a strong correlation between increased IsoP levels and vascular dysfunction. These data suggest a role for superoxide and its coupling product formed with nitric oxide, peroxynitrite, in lipoprotein oxidation and vascular function *in vivo*.

Organophosphate poisoning is associated with muscle endplate necrosis and increased levels of IsoPs esterified to muscle tissue were recently demonstrated in animals poisoned with organophosphates.[77] Further, administration of a lazaroid antioxidant suppressed both levels of IsoPs and protected against organophosphate-induced muscle necrosis, suggesting that free radicals are involved in the pathologic changes that occur in the muscle in association with organophosphate poisoning.

Increased formation of IsoPs has also been demonstrated in settings of ischemia/reperfusion injury to both the liver and kidney.[44,78] Further, dietary iron overload has been shown to be associated with increased levels of F_2-IsoPs esterified to lipids in the livers of rats.[79] The anesthetic halothane can induce liver injury, especially under hypoxic conditions, which is thought to involve the production of free radicals via the reductive metabolism of halothane.[80] We recently demonstrated that in rats given halothane, even under normoxic conditions, increased levels of F_2-IsoPs are present esterified to hepatic lipids, indicative of free radical induced peroxidation of hepatic lipids.[81]

D. Quantification of F_2-isoprostanes to Assess the Role of Oxidant Injury in Human Diseases

From the above examples, measurement of IsoPs appears to be a reliable index of lipid peroxidation *in vivo* and thus potentially provides us with a tool to assess the role of free radicals in the pathophysiology of human disease. Some examples where measurement of IsoPs has provided new evidence for a role of oxidant stress in human disease are summarized below.

1. *Hepatorenal Syndrome and Acetaminophen Poisoning*

We have explored the hypothesis that oxidant stress may play a role in the hepatorenal syndrome. Hepatorenal syndrome is defined as the onset of renal failure of unknown etiology in patients with severe liver disease. The pathophysiology of the renal failure may

be due to intense renal vasoconstriction but the cause of the renal vasoconstriction remains poorly understood.[82] These patients often exhibit chronic endotoxemia and tissue hypoxia, an environment conducive for the generation of free radicals. Therefore, we quantified circulating levels of F_2-IsoPs in 12 patients with the hepatorenal syndrome and appropriate control groups including normal volunteers, patients with chronic renal failure and normal liver function, and patients with severe and mild liver disease in whom renal function was normal.[83] Circulating plasma concentrations of F_2-IsoPs were selectively increased a mean of 7.8-fold in patients with hepatorenal syndrome compared with the control groups ($p < 0.001$). We also measured circulating concentrations of F_2-IsoPs in 10 patients with acute liver and renal failure associated with acetaminophen (paracetamol) overdose. In this group, plasma F_2-IsoP concentrations were increased a mean of 9.1-fold above normal ($p < 0.001$). Whether the renal failure in these patients is due to direct nephrotoxicity from acetaminophen metabolites[84] and/or a variant of the "conventional" form of hepatorenal syndrome is not known. Further, in three of these patients we examined the effect of infusing a single dose of superoxide dismutase on circulating levels of F_2-IsoPs. In all three patients, plasma levels decreased to $\sim 50\%$ of preinfusion levels between 30 and 60 min after administration of the agent, consistent with a oxygen centered radical process. Since the duration of the effect of the administration of a single dose of superoxide dismutase is short due to its rapid elimination, the effect of the reduction of plasma concentrations of F_2-isoprostanes on renal function could not be assessed in this acute study.

These findings suggest a role for free radicals in the pathogenesis of this almost uniformly fatal disease. It is noteworthy that liver transplantation in these patients is often associated with a return of normal renal function.[85] Unfortunately, many patients with hepatorenal syndrome die before a donor liver can be found. However, our results form a rational basis to explore whether antioxidant therapy may be effective in preventing death during the interval between the onset of hepatorenal syndrome and identification of a suitable donor liver. As previously discussed, both 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) and 8-iso-PGE_2 (15-E_{2t}-IsoP) are potent renal vasoconstrictors. Although it remains to be proven, it is attractive to consider the possiblity that these IsoPs are contributing to the renal vasoconstriction that characterizes this disorder. In addition, the finding that 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP) induces endothelin-1 release may help to explain the large increases in plasma endothelin-1 concentrations in the hepatorenal syndrome.[86] As noted previously, the vasoconstriction caused by these IsoPs can be diminished by at least some thromboxane receptor antagonists. In this regard, we have a study currently underway to assess whether treatment of patients with hepatorenal syndrome with a thromboxane receptor anatagonist is associated with an improvement in renal function; preliminary results from this study appear promising.

2. *Scleroderma*

The pathogenesis of scleroderma (systemic sclerosis) is unknown but it has been proposed that oxidant injury is involved in the tissue damage associated with this disorder.[87] Not only can free radicals stimulate fibroblast proliferation and fibrosis, which characterize this disease, they can also inhibit the action of NO resulting in enhanced vasoconstriction, which is another hallmark of scleroderma. To examine whether there was evidence of oxidative stress in scleroderma, we measured the excretion of F_2-IsoP's urinary metabolites in 10 healthy controls and eight patients with scleroderma (representing a wide spectrum of disease severity ranging from limited to diffuse disease).[88] The urinary excretion of F_2-IsoP metabolites was elevated in scleroderma patients to a mean of 2.8-fold above control ($p < 0.002$). Elevations were seen both in patients with limited and diffuse disease but the highest excretion of F_2-IP metabolites (483% above control) was seen in a patient with severe diffuse disease.

These data suggest a role for free radicals in the pathogenesis of scleroderma and provide a marker whose relationship to disease activity and disease therapy may be important.

Further, these findings may also provide a rationale to explore whether antioxidant therapy may influence the natural course of this disease.

3. *Chronic Cigarette Smoking*

Cigarette smoke contains a number of free radicals and oxidants and it has been suggested that cigarette smoking may cause oxidative damage.[89] Since oxidation of DNA and LDL may lead to cancer and atherosclerosis, respectively, oxidative injury to these important biomolecules by cigarette smoke may provide a mechanistic link between the enhanced incidence of cancer and atherogenesis in individuals who smoke.[15] To determine whether there is increased oxidative stress in smokers, we measured levels of F_2-IsoPs both free in the circulation and esterified to plasma lipids and the urinary excretion of F_2-IsoP metabolites in 10 heavy smokers and 10 carefully selected age- and gender-matched non-smokers. Levels of both free and esterified F_2-IsoPs were significantly elevated in smokers compared to non-smokers by a mean of 235% ($p = 0.02$) and 166% ($p = 0.03$), respectively.[15] Further, there was a high correlation between the urinary excretion of F_2-IsoP metabolites and plasma concentrations of F_2-IsoPs ($r = 0.97$, $p < 0.001$). Following 2 weeks of abstinence from smoking, levels of free and esterified F_2-IsoPs in plasma fell significantly by a mean of approximately 35%. Recently, Fitzgerald and colleagues have also reported increases in urinary levels of the IsoP, 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP), in smokers and have found that levels decrease significantly after administration of the antioxidant vitamin C.[90] These findings suggest that cigarette smoking causes oxidative injury and this may provide a causative link between smoking and the development of cancer and atherosclerosis.

4. *Other Disorders*

Enhanced production of IsoPs has also been reported to occur in association with several other disorders in which free radicals have been thought to play a role. Levels of IsoPs are increased in tracheobronchial aspirate fluid of premature infants exposed to increased oxygen concentrations (unpublished observations) and in tracheal lavage fluid from human adults exposed to ozone.[91] There also seems to be a trend towards increased production of IsoPs with increasing age, data which may lend some support to the oxidation hypothesis of aging.[31] In addition, a recent report also notes an increase in circulating levels of IsoPs in individuals with diabetes.[92] The administration of cyclosporine is frequently associated with a decrease in renal function, which has been speculated to involve free radical injury to the kidney.[93] However, we recently demonstrated that alterations in renal function in patients with rheumatoid arthritis being treated with cyclosporine were not associated with an increase in urinary IsoPs, suggesting that cyclosporine, at the doses administered to these patients, does not induce lipid peroxidation.[94]

XI. SUMMARY

The discovery of IsoPs as products of non-enzymatic lipid peroxidation has opened up new areas of investigation regarding the role of free radicals in human physiology and pathophysiology. The quantification of IsoPs as markers of oxidative stress status appears to be an important advance in our ability to explore the role of free radicals in the pathogenesis of human disease. A drawback related to this, however, has been lack of more facile and less expensive methods than mass spectrometry for the measurement of IsoPs. On the other hand, the recent introduction of immunoassay methods for measurement of IsoPs may alleviate this problem, provided they are specific and reliable. If this is the case, immunoassay methodology will most likely lead to an expansion of the use of measurements of IsoPs to assess oxidative stress status *in vivo*. Another need in the field of free radical medicine is information regarding the clinical pharmacology of antioxidant agents. Because of the evidence implicating free radicals in the pathogenesis of a number

of human diseases, large clinical trials are planned or underway to assess whether antioxidants can either prevent the development or ameliorate the pathology of certain human disorders. However, data regarding the most effective doses and combination of antioxidant agents to use in these clinical trials is lacking. As mentioned previously, administration of antioxidants suppresses the formation of IsoPs, even in normal individuals. Thus, measurement of IsoPs may provide a valuable approach to defining the clinical pharmacology of antioxidants.

In addition to being markers of oxidative stress, at least two IsoPs possess potent biological activity. The availability of additional IsoPs in synthetic form should broaden our knowledge concerning the role of these molecules as mediators of oxidant stress. Moreover, information regarding the nature of the receptor(s) that mediate the biological actions of IsoPs will be of considerable importance to the development of specific antagonists or agonists of the biological actions of IsoPs.

Despite the fact that considerable information has been obtained since the initial report of the discovery of IsoPs,[6] much remains to be understood about these molecules. With continued research in this area, we believe that much new information will emerge that will open up additional important new areas for future investigation.

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